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14. ABSTRACT

Rate of prostate cancer progression is affected by the reactive stroma microenvironment. Our previous studies have shown that reactive stroma is regulated by TGF-β pathways. The objectives of the proposed research is to assess the origin / ontogeny of reactive stroma in cancer and the fundamental mechanisms of recruitment / activation in prostate cancer. To date, we have addressed studies proposed for each Task. We have developed an in vivo matrix trapping approach to isolate, characterize and culture reactive stromal cells recruited to Matrigel plugs. We have also developed a three dimensional co-culture model that permits co-culture of prostate carcinoma cell spheroids with prostate stromal progenitor cells. We have evaluated reactive stroma recruitment in mice that receive xenograft implants of prostate cancer cells. We have assessed the effects of TGF-β on reactive stroma differentiation. These studies will allow us to dissect key mechanisms that mediate recruitment of reactive stroma to the tumor microenvironment and to target these mechanisms in order to inhibit tumorigenesis.

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Introduction:

Reactive stroma in the immediate cancer microenvironment is a key regulator of prostate cancer progression. Mechanisms and specific reactive stroma progenitor cells are unknown. Our laboratory has made considerable progress in evaluating the key cell phenotypes in reactive stroma [1, 2]. Our most recent studies have attempted to identify the reactive stroma stem/progenitor cells and the mechanisms responsible for their activation to reactive stroma myofibroblasts. Our studies indicate that reactive stroma forms at foci of prostatic intraepithelial neoplasia and that transforming growth factor beta (TGF-β) and interleukin 8 (IL-8) may each play a role in recruiting and/or activating reactive stroma cells [3-5]. We have made considerable progress in collaborative studies and have reported that reactive stroma is tumor promoting and can be used as a predictor of prostate cancer progression to biochemical recurrent disease [6, 7] and as a predictor of those patients that will actually die from their prostate cancer[8]. In these collaborative studies, we have also shown that nerves regulate prostate cancer progression. Accordingly, the tumor microenvironment exhibits a complex regulatory biology that is a key regulator of prostate cancer progression. Since the responses of the reactive stroma compartment is predictable in prostate cancer, the targeting of reactive stroma or reactive stroma stem/progenitor cells is emerging as a potentially new therapeutic approach. The immediate goals of this research is to establish whether reactive stroma recruitment to the cancer foci can be used as a novel therapeutic targeting point. The long-range goal is to target reactive stroma recruitment as a novel therapeutic approach to disrupt the biology of the reactive stroma niche and therefore alter the progression of the cancer.

Body:

Task 1, Task 1 was to complete the proposed Specific Aim 1 studies as follows: "To determine mechanisms of local reactive stroma recruitment using a novel matrix trapping procedure and to assess the role of TGF-β in driving both recruitment and activation to reactive stroma phenotype."

The goal of Task1 (Aim 1) is to define the population of cells recruited to reactive stroma. We initiated these studies with a novel matrix trapping approach and have developed a new three dimensional (3D) organoid co-culture approach. These approach have permitted us to identify reactive stroma progenitor (RSP) cells in the human prostate gland. The 3D co-culture organoid model is being used to dissect the key mechanisms in differentiation of RSP cells to reactive stroma myofibroblasts.

Most of the goals of this Task/Aim have been completed. The initial matrix trapping studies served to culture trapped stromal cells as we reported in the 2010 Annual Report. The trapped cells have been subpassaged and frozen. We are planning experiments to assess the RSP status of these cells using the 3D organoid approach to complete the Task/Aim.

To address human reactive stroma, we have characterized the HPS-19I cells as putative RSP cells using the 3D organoid model as discussed in the 2010 Annual Report. This cell line was

initiated from normal human cadaver prostate tissue with no histopathological evidence of prostate disease. The cell line was continuously cultured in media Bfs following protocols we have published previously [9-12]. These cells are CD44+. CD90+, CD133+ and express other markers of mesenchymal stem cells. The cells exhibit a high self-renewal potential, are diploid, and have been passaged for more that 40 passages. They do not exhibit pluripotent differentiation and are restricted to differentiation to only reactive stroma myofibroblasts, based on our studies to date.

The 3D organoid co-culture model was developed as shown in Figure 1. This model uses the 12 mm diameter Millicell-CM membrane (0.4 μm pore) insert (Millipore) in 24 well tissue culture plate fitted with 12 mm diameter PolyD-Lysince/Laminin Cellware coated glass cover slips (BD BioCoat). LNCaP human prostate cancer cells are seeded in the top chamber either alone or with HPS-19I RSP cells. The bottom well is either left unseeded or receives HPS-19I RSP cells. Hence, this approach is flexible and can examine the effects of co-culture when cells are in direct contact (both cells in upper chamber) or not in contact (LNCaP in upper and HPS-19I in bottom well). Since the CM membrane does not permit cell attachment, the cells self attach as form 3D spheroids in the upper chamber. Cultures are seeded in serum-contaning media and switched to serum-free defined media for 72 hr. Remarkably, mixed cultures self-organize with HPS-19I stromal cells in the interior with an exterior mantel of LNCaP cells (Figure 2).

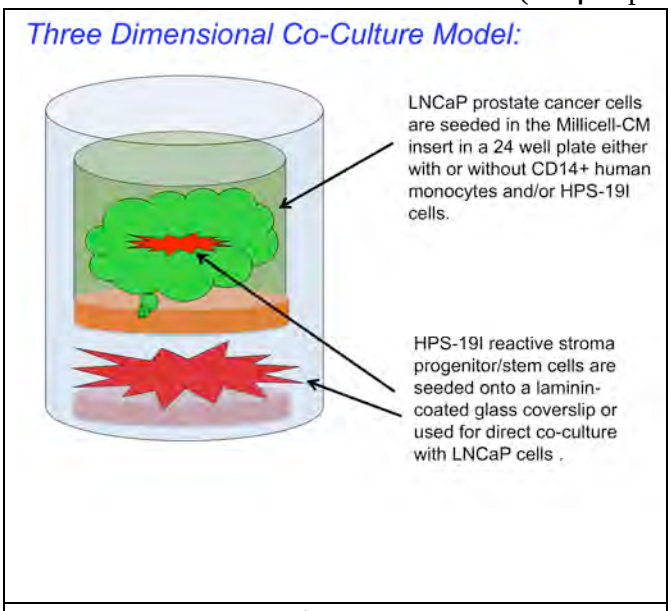

Figure 1. Generation of a three-dimensional co-culture system. Millicell-CM inserts are placed in 24 well plates that contain a laminin-coated glass coverslip. LNCaP organoids are cultured in the upper insert and putative stromal progenitor/stem cells are cultured on the coverslip in the lower compartment.

To summarize Task1/Aim 1, we have made considerable progress in designing and using the 3D organoid approach and now have a well controlled, serum-free, recombined model to assess key factors that regulate RSP cell activation to reactive stroma cells. We anticipate studies will pinpoint the key transcription factors and growth factors that mediate this inductive process. Moreover, as a part of another project, not proposed in the present application, we will examine the influence of reactive stroma on the expression of androgen-regulated genes in LNCaP cells.

Task 2, Task 2 is to complete Specific Aim 2 studies: To determine the mechanisms of reactive stroma recruitment from marrow-derived circulating progenitors and to assess the role of TGF-β in active recruitment and induced differentiation to reactive stroma.

Adoptive transfer experiments and bone marrow transplant studies have been completed and were discussed in the 2010 Annual Report. We have also completed other studies to isolate bone

marrow derived cells from the eGFP mice. These have been isolated, cultured and frozen. We anticipate using these cells in the 3D organoid model to again assess the ability of prostate cancer cells that overexpress active TGF-β1 to induce reactive stroma.

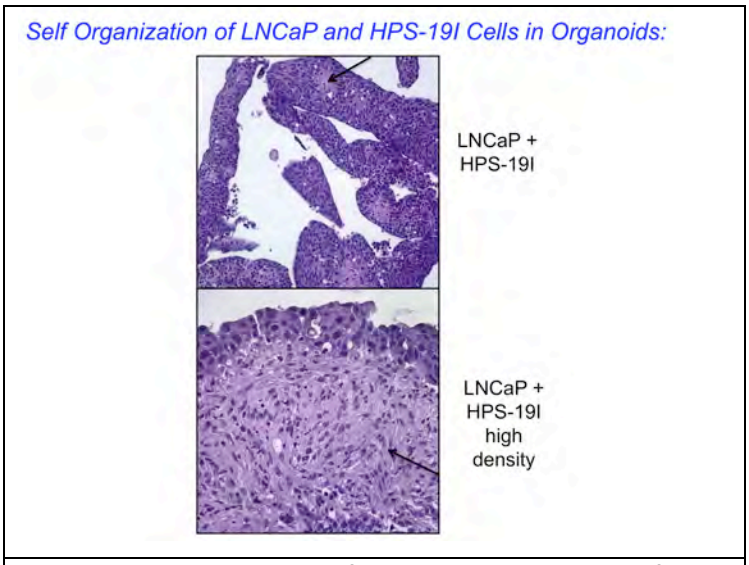

Figure 2. 3D organoids self organize with a mantel of LNCaP cells and a center core of HPS-19I reactive stroma progenitor cells (arrows).

We have concluded from the marrow transplants studies that bone marrow derived cells are indeed recruited to sites of developing reactive stroma. These recruited cells seem to differentiate to monocyte-like or myeloid-like cells based on phenotypic appearance. They appear as dendritic-like cells as well, however, we have not yet completed an exhaustive study of phenotypic differentiation. They do not likely differentiate to myofibroblasts or carcinoma-associated fibroblasts.

To address the role of TGF-β1, we engineered LNCaP cells to overexpress constitutively active TGF-β1 and used these in the 3D organoid model. Using this model system, we were able to show during the last progress period that several growth factors and transcription factors are upregulated in HPS-19I cells when they are co-cultured on the laminin coated coverslips with LNCaP cell organoids in the upper chamber engineered to express constitutively active TGF-β1 (Figure 3). Moreover, when cells were recombined (both in the upper chamber) these conditions resulted in differentiation of HPS-19I cells into prototypical myofibroblasts that express both tenascin-C and smooth muscle α-actin. (Figure 4). Together, these studies show that prostate cancer cells that overexpress TGF-β1 results in the activation of RSP cells to become prototypical reactive stroma myofibroblasts.

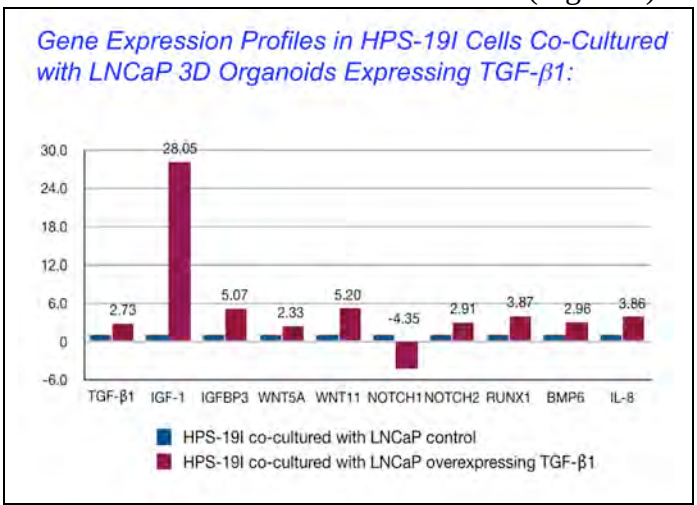

Figure 3. Differential gene expression in HPS-19I cells co-cultured with LNCaP cells that express active TGF-β1.

To summarize progress on Task 2/Aim 2, we have shown that reactive stroma myofibroblasts are most likely derived from an endogenous reactive stroma progenitor cell that is activated by TGF-β1 expressed by adjacent prostate cancer cells. These studies suggest that this endogenous RSP cell is the target of opportunity in trying to manipulate the cells of origin for forming reactive stroma. This was a key goal of this project. Our future studies are focused now on

dissecting key mechanisms, as each of these pathways are potential targets for new therapeutics.

Task 3. Task 3 will be to complete Specific Aim 3 studies: To use a drug-inducible expression system to assess whether progenitor cells can be targeted to deliver drug-induced gene expression at sites of recruitment / activation of reactive stroma and whether this approach can uncouple key recruitment pathways.

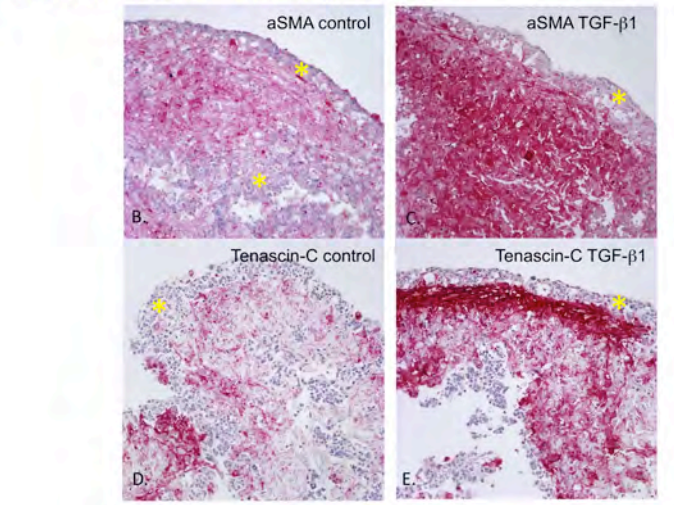

Figure 4. HPS-19I cells differentiate to prototypical myofibroblasts when placed in 3D organoid culture with LNCaP cells overexpressing active TGF-β1. HPS-19 I express smooth muscle a-actin (a-SMA) (panel C) and tenascin-C (panel E) when they differentiate to reactive stroma.

Our current plan is to use the pIRSUE lentivirus system to engineer HPS-19I cells. This permits doxycycline induced gene expression. The pIRSUE system is designed to express doxycycline induced shRNA expression. We are currently engineering HPS-19I cells to express shRNA to genes we think are most relevant to the TGF-β1 induced reactive stroma phenotype. This includes the transcription factors RUNX1 and ID1. Key growth factors we are currently addressing is IGF-1. Progress has been made on each of these. We plan on using these cells in the 3D organoid approach and then will proceed to in vivo studies that will use the DRS xenograft system as we have described in our earlier studies.

Key Research Accomplishments:

- Development of an *in vivo* matrix trapping procedure that allows for phenotype characterization of recruited reactive stroma in situ and for isolation of these cells for cell culture.

- Development of a first generation *in vitro* co-culture method that uses LNCaP spheroids combined with prostate stromal cells in fully defined and serum-free culture media.

- Development of protocols to isolate and culture circulating murine pro-fibrocyte cells, murine bone marrow stromal cells and human CD14+ monocytes.

- Development of a second-generation *in vitro* and three-dimensional co-culture method that permits modular inclusion of several cell types in direct contact with LNCaP 3D organoids or in a separate chamber and cultured on coverslips with different attachment factors in fully defined media.

- Demonstration of key changes in putative stromal progenitor/stem cell differentiation and gene expression profiles that is affected by both prostate cancer cells and CD14+ monocytes.

- Demonstration that recruitment of bone marrow derived cells to LNCaP xenografts using adoptive transfer and bone marrow transplant studies. These studies suggest that these cells are monocyte-like cells that do not differentiate to reactive stroma myofibroblasts or carcinoma-associated fibroblasts.

- Key data that suggests reactive stroma is likely derived from resident stromal progenitor/stem cells that are influenced to differentiate via interactions with cancer cells and recruited bone marrow derived monocyte/macrophages. These data help to pinpoint these cells as the targets for drug-induced gene expression to be done in Task 3 studies.

- Definitive data showing that overexpression of TGF-β1 in prostate cancer cells induces differentiation of reactive stroma progentior cells to prototypical reactive stroma myofibroblasts.

- Data that profiles the gene expression of reactive stroma progenitor cells during their activation by prostate cancer cells that overexpress active TGF-β1.

Reportable Outcomes:

- Presentation of this data at several seminars and national meetings (including the recent IMPACT meeting) by Dr. Rowley.

- Some of this data was used to support the proposed use of the co-culture methodology in several other research proposals. These have been submitted to the NIH, the DOD and to the Cancer Prevention Research Institute of Texas (CPRIT).

Conclusions:

Considerable progress has been made on on Tasks 1 and 2 and these studies have reached their natural endpoints. We had planned to prepare and submit a manuscript during the last progress period, however, we wanted to more fully characterize gene expression data for this study. We are now close to being able to submit the manuscript on these data. We anticipate Task 3 to be completed in the next 6-9 months. This work has led to development of a novel co-culture method that recombines human prostate carcinoma cell spheroid/organoids with human prostate stromal progenitor/stem cells in a three dimensional culture model that is maintained in serum-free conditions. We think this approach will allow us to dissect key mechanisms important for reactive stroma recruitment and activation. This is the goal of these studies. We should be able to target cells with inducible gene expression as a proof of concept that targeting the reactive stroma microenvironment is possible. It is anticipated that these studies will allow us to develop critical pre-clinical data from which to base a strategic approach aimed at targeting the reactive stroma in human patients.

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